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Mechanism of the interferon alpha response against hepatitis C virus replicons

Ju-Tao Guo, Ji A. Sohn, Qing Zhu, and Christoph Seeger*

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

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Abstract

Interferon alpha (IFN-α) inhibits hepatitis C virus (HCV) replication in vivo and in cell cultures by one or several mechanisms that are not yet understood. We sought to identify the viral targets of the IFN-α-induced cellular antiviral program in Huh7 cells expressing HCV subgenomic replicons. Our results revealed a tight linkage between translation, assembly of replication complexes and viral RNA synthesis, and indicated that the stability of amplified plus strand RNA was reduced in the presence of the cytokine. Moreover, HCV internal ribosomal entry site (IRES)-directed translation was inhibited approximately 2-fold in IFN-treated cells. In contrast, the synthesis of viral RNA did not seem to be directly affected by the antiviral program induced by the cytokine. Our results were consistent with a model predicting that the IFN-α-induced antiviral program could inhibit multiple steps of the HCV replication cycle, leading to a reduction in viral protein synthesis and eventually inhibition of viral RNA amplification.

Keywords: IFN-α; Hepatitis C virus; RNA amplification

Introduction

Hepatitis C virus (HCV) infections affect the health of more than 170 million people worldwide and are currently the most common basis for liver disease in the United States (Alter and Seeff, 2000). HCV is a member of the family *Flaviviridae*, which also includes some well-known human and animal pathogens, such as yellow fever virus, West Nile virus, Dengue virus and bovine viral diarrhea virus (Choo et al., 1989). These viruses have in common a single-stranded, positive-sense RNA genome carrying one long open reading frame that is flanked by non-translated regions (NTR). The HCV genome has a length of about 9600 nucleotides and encodes an approximately 3000-amino acid-long polyprotein that is proteolytically processed into 10 polypeptides (Reed and Rice, 2000). Three are structural proteins required for capsid formation (core) and assembly into enveloped viral particles (E1 and E2). Four virus-encoded proteins are enzymes including cysteine and serine proteases (NS2 and NS3), an ATP-dependent helicase (NS3) and a RNA-directed RNA polymerase (NS5B). The functions of the remaining three polypeptides, p7, NS4B and NS5A, for viral replication are not yet known. The 5′ NTR spans about 340 nucleotides and harbors the *cis*-elements for viral RNA replication and an internal ribosomal entry site (IRES) directing the translation of the viral polyprotein. The 3′ NTR has a highly conserved sequence element (3′ X) that is essential for viral RNA replication.

The model for the HCV life cycle predicts that replication begins with the translation of the polyprotein following infection of permissive cells. Accumulation of viral proteins induces rearrangements of ER membranes that form the locales for replication of viral RNA (Egger et al., 2002; Westaway et al., 1997). Minus strand RNA synthesis leads to the formation of a double-stranded RNA molecule that bears a promoter at the 3′ end of minus strand RNA required for amplification of plus strands by a semiconservative mechanism (Chu and Westaway, 1985). As a consequence of this mechanism, the concentrations of plus and minus strands differ approximately 10-fold (Chu and West-

* Corresponding author. Institute for Cancer Research, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111. Fax: +1-215-728-4329.
E-mail address: seeger@fccc.edu (C. Seeger).

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away, 1985). The fate of plus strands is 2-fold. Early in infection, they are transported to ribosomes for additional rounds of translation and replication of viral RNA. Late in infection, they are packaged for the production of virus particles. The mechanism controlling the fate of plus strands is not yet understood.

Despite several reports claiming that HCV can infect cultured cells, the levels of viral replication were generally too low to allow for detailed analyses of viral replication (i.e. Shimizu et al., 1992; Sung et al., 2003). The development of selectable subgenomic replicons facilitated the study of viral replication in cultured cells and provided a cell-based assay for the screening and testing of drugs that inhibit viral replication (Lohmann et al., 1999). Subgenomic replicons carry in lieu of the structural genes the gene encoding neomycin phosphotransferase II (NPT II). An IRES derived from EMCV directs the translation of the non-structural proteins. Replication of HCV replicons was first demonstrated in the human hepatoma cell line Huh7, and more recently, in HeLa and mouse hepatoma cells (Blight et al., 2000; Lohmann et al., 1999; Zhu et al., 2003). Due to the lack of structural genes, packaging of plus strand RNA and assembly of virions cannot occur in the HCV replicon system and hence, amplified RNA is either reused for translation or is degraded by cellular ribonucleases.

HCV replication can be inhibited by interferon alpha (IFN-α) in infected patients and by addition to the medium of Huh7 cells expressing subgenomic replicons or full-length genomes (Blight et al., 2000; Frese et al., 2001; Guo et al., 2001; Neumann et al., 1998). In vivo, the potency of the cytokine varies significantly depending on the genotype of the virus and on many other factors that have not yet been identified (Di Bisceglie and Hoofnagle, 2002; Kinzie et al., 2001; McHutchison et al., 1998). IFN-α induces about 300 genes that alter cell homeostasis and can lead to programmed cell death of infected cells or inhibition of viral replication (Der et al., 1998; Sen, 2001; Stark et al., 1998). A recent study with tissue culture cells revealed that IFN-α can induce cytopathic and non-cytopathic responses in cells expressing HCV replicons (Guo et al., 2003). However, the cellular proteins inhibiting HCV replication and the primary target of IFN-induced antiviral proteins in the HCV replication cycle have not yet been identified. Several studies intended to determine whether IFN-α blocks translation of HCV proteins provided conflicting results, likely reflecting differences among the experimental systems used for those analyses (Kato et al., 2002; Koev et al., 2002; Shimazaki et al., 2002; Wang et al., 2003).

To gain a better understanding of the mechanism by which IFN-α inhibits HCV replication, we sought to identify the viral target(s) of the antiviral program elicited by the cytokine in Huh7 cells. Specifically, we investigated whether translation, replication complex formation or RNA amplification were inhibited by IFN-α.

Results and discussion

HCV RNA replication and viral protein synthesis are tightly linked events

Consistent with previous reports, IFN-α treatment of Huh7 cells expressing subgenomic replicons, such as the cell line GS4.1, led to a rapid reduction of viral RNA levels and, simultaneously, led to an inhibition of viral protein synthesis (Figs. 1A–C). Inhibition of NPT II and NS5A synthesis, as measured with ^{35}S-methionine and ^{35}S-cysteine pulse labeling experiments, occurred with similar efficiency, indicating that translation from both, the HCV and EMCV IRES elements, was affected in a similar manner (Fig. 1D). Under the same conditions, the translation of cellular proteins was not affected by IFN-α (results not shown).

The observed inhibition of translation by IFN-α could have been caused either directly by inhibition of IRES-directed translation or indirectly, as a consequence of the reduction of viral plus strand RNA levels. To distinguish between these two possibilities, we took advantage of the nucleoside analogue 2′-C-methyl adenosine (2CMA) that was previously shown to be an inhibitor of HCV replication in cells with an IC_{50} = 0.2 μM. (Carroll et al., 2003). Incubation of GS4.1 cells with 5 μM 2CMA reduced viral RNA levels with similar kinetics observed following incubation with 100 IU/ml IFN-α (Fig. 1). In contrast, viral protein synthesis was more strongly inhibited by 2CMA than by IFN-α. Notably, the results revealed that 6 h after incubation of cells with the polymerase inhibitor, RNA levels decreased by only 20% compared with an 80% reduction in viral protein synthesis. Similar results were obtained with two other experiments that were carried out under similar conditions (results not shown).

These results indicated that translation of viral RNA was dependent on continuous RNA synthesis and suggested that only a fraction of plus strands present in cells was used for the translation of viral proteins. As a consequence of such a tight linkage between RNA synthesis and protein translation, it was not possible to directly determine the effects of IFN-α on HCV translation in a stable replicon cell line.

Effects of IFN-α on HCV IRES-directed translation

To directly measure the effects of IFN-α on IRES-directed translation, we transfected Huh7 cells with the dicistronic reporter plasmids, pHCVneo3 and pEMCVneo6 (Fig. 2A). Pools of transfected cells were incubated for 24 h with different amounts of the cytokine and the levels of cap- and IRES-dependent translation were determined. The results showed that at concentrations of IFN-α known to inhibit HCV RNA replication (10 IU/ml), translation of NPT II from the EMCV or HCV IRES elements was not reduced (Fig. 2C). Even at concentrations that exceeded the IC_{50} of IFN-α (ca. 1 IU/ml) by 1000-fold, no inhibition of

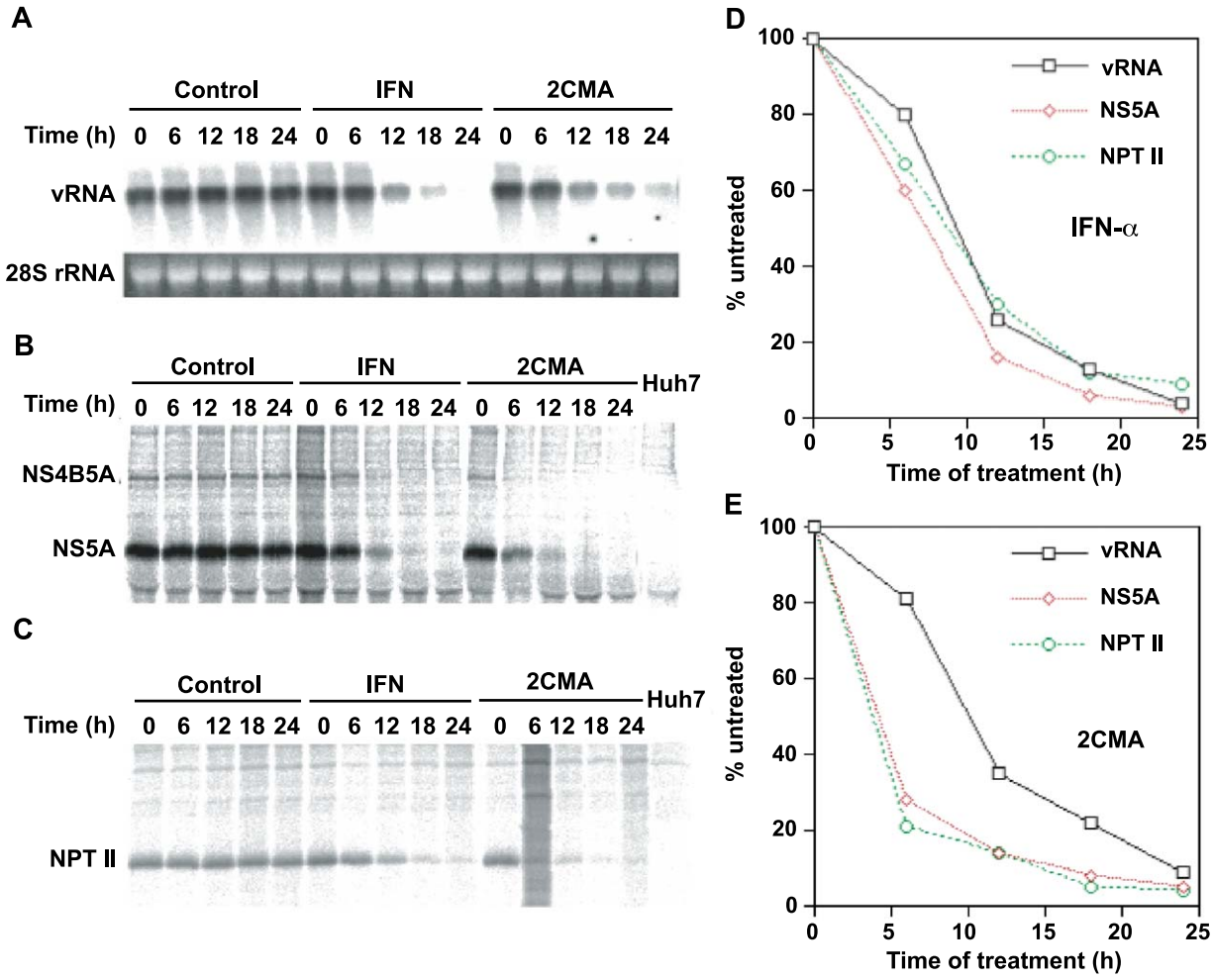

Fig. 1. HCV RNA levels and protein synthesis in IFN-α- and 2CMA-treated GS4.1 cells. (A) GS4.1 cells were treated with 100 IU/ml IFN-α or 5 μM 2CMA. Cells were harvested before treatment (0) and 6, 12, 18 and 24 h posttreatment. Viral RNA levels were determined by Northern blot analysis. Ribosomal 28S RNA was used as a control for the amount of RNA loaded in each lane. (B and C) GS4.1 cells were cultured in medium alone (control), or medium containing 100 IU/ml IFN-α or 5 μM 2CMA for the indicated time periods. Cells were then pulse-labeled with Express Labeling Mix (NEN) for 2 h. NS5A and NPT II synthesis were determined by immunoprecipitation assay. (D and E) The amount of viral RNA, NS5A and NPT II were measured with a phosphoimager and plotted as the fraction (%) of the values obtained with untreated cells.

IRES-dependent translation could be observed. The reason for the appearance of the second, faster migrating form of NPT II is not known. A weak reduction in cap-dependent synthesis of DHBsAg was apparent at the highest concentrations (1000 IU/ml) of the cytokine used for these experiments (Fig. 2B).

Although these results could be interpreted to mean that IFN-α did not inhibit HCV IRES-directed translation, our experiments did not preclude the possibility that differences existed between di-cistronic mRNAs and subgenomic HCV RNA for translation. For example, it is conceivable that the proposed interaction between the 5′ and 3′ NTRs could play a role in the regulation of IRES-mediated translation (Khromykh et al., 2001). To resolve this problem, we transfected IFN-α-treated and mock-treated Huh7 cells with in vitro transcribed subgenomic replicon RNAs and determined the levels of NPT II translation, which is directed by the HCV IRES. To distinguish between translation from replicated RNA and input RNA, we transfected cells with replication-competent (pZS11) and -deficient (pZS2N) RNAs (Fig. 3). Translation of NPT II was determined by pulse labeling with ^{35}S-methionine and ^{35}S-cysteine following the electroporation of Huh7 cells that were pretreated with IFN-α for 6 and 12 h, respectively (see Materials and methods). The results from four experiments showed that IFN-α reduced the translation of NPT II from pZS11 and pZS2N replicon RNA to 40–60% of the levels in untreated Huh7 cells (Figs. 3A and B). The small differences observed between pZS11 and pZS2N are most likely explained by amplification of input RNA in case of the former, but not the latter.

From these results, we concluded that inhibition of translation could have contributed to the antiviral effects of IFN-α on HCV replicons in Huh7 cells, but that it was too weak to explain the antiviral activity of the cytokine. This view was supported by the observation that RNA replication could continue in the presence of cycloheximide (CHX), and

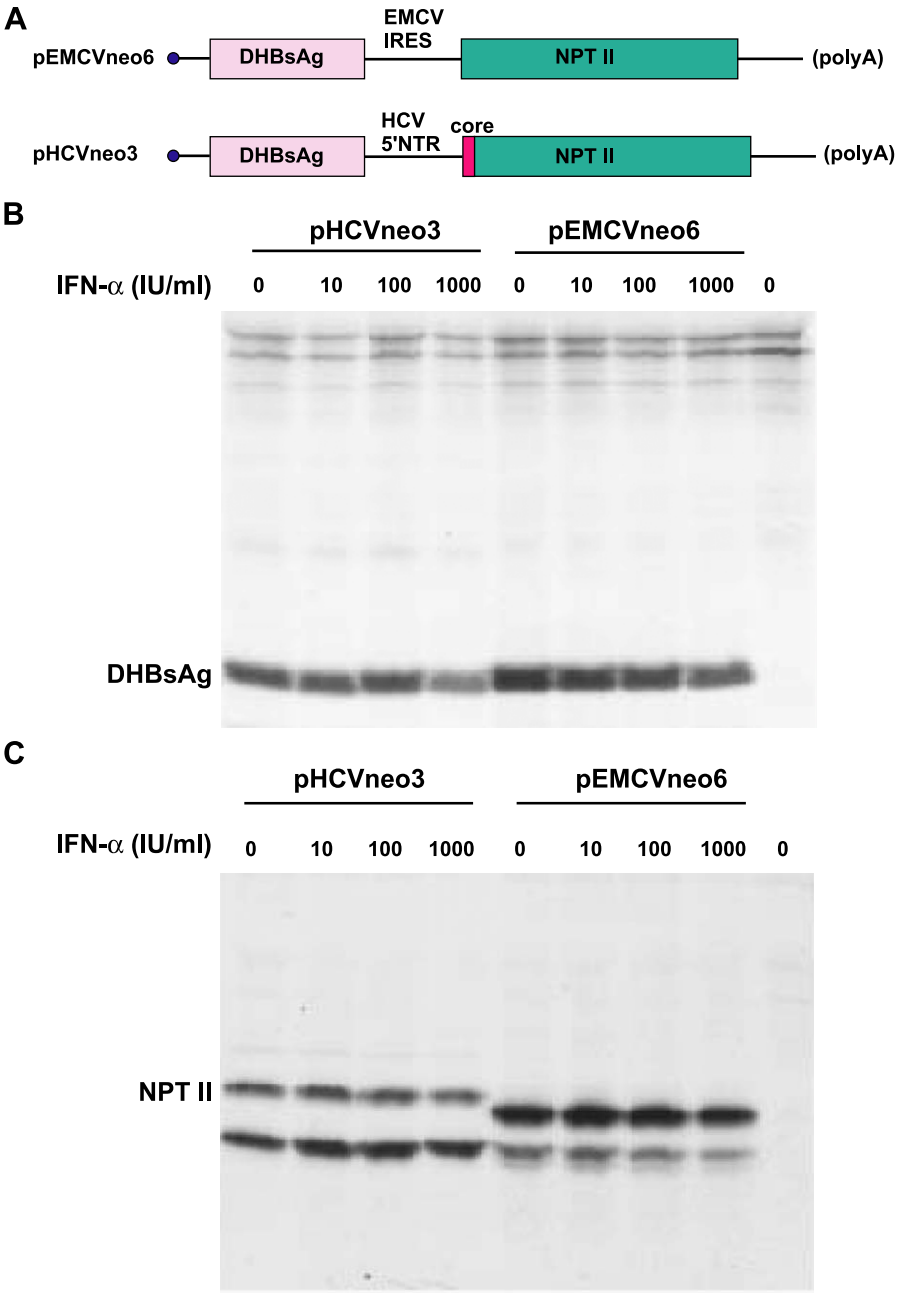

Fig. 2. Effects of IFN-α on HCV and EMCV IRES-directed translation in Huh7 cells. (A) Schematic diagrams of bicistronic mRNA. Plasmid pEMCVneo6 encodes a transcript containing the surface antigen gene of duck hepatitis B virus (DHBsAg), EMCV IRES and NPT II gene. Plasmid pHCVneo3 encodes a transcript containing the surface antigen gene of duck hepatitis B virus (DHBsAg) fused to the HCV IRES (5′NTR) including 36 nt from the core gene and the complete NPT II gene. In both cases, DHBsAg translation depends on cap-dependent mechanism, but NPT II translations depend on EMCV and HCV IRES, respectively. (B and C) Huh7 cells were transfected with plasmids pHCVneo3 and pEMCVneo6 by calcium phosphate precipitation procedure (Clontech Laboratories, Inc.). Forty-eight hours posttransfection, cells were mock-treated or treated with the indicated concentrations of IFN-α for 24 h. Cells were then pulse-labeled with Express Labeling Mix (NEN) for 2 h. The synthesis of DHBsAg and neomycin NPT II were determined by immunoprecipitation assay.

that under those conditions, the rate by which positive strand RNA declined was much slower than observed with IFN-α (Fig. 4). The apparent $t_{1/2}$ of viral RNA in the presence of the antibiotic was about 20 h, more than twice the time determined for viral RNA in the presence of IFN-α or 2CMA (Figs. 4B and 6D). Moreover, results from metabolic labeling of viral RNA with ^{32}P-orthophosphate confirmed that viral RNA synthesis could continue for some time in the absence of translation and that the inhibition of viral RNA synthesis by CHX was weaker than observed with IFN-α (Fig. 5). Those results were consistent with results obtained from similar experiments with Kunjin virus (Westaway et al., 1999). Taken together, our results indicated that besides the inhibition of viral protein translation, the IFN-α induced antiviral program could also target other steps of the viral replication cycle, such as viral RNA synthesis, transfer of de

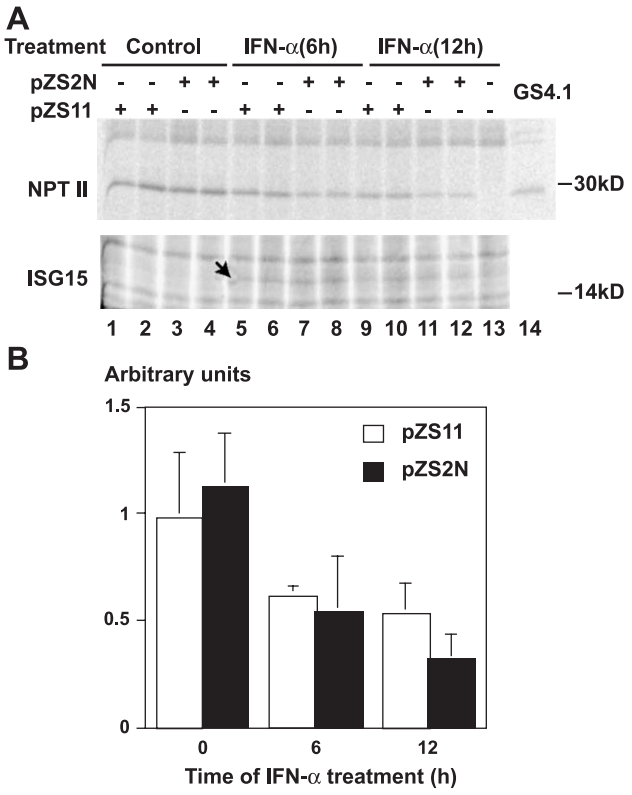

Fig. 3. Effects of IFN-α on HCV IRES-directed translation in Huh7 cells. (A) Huh7 cells were left untreated (control) or treated with 100 IU/ml of IFN-α for 6 and 12 h, respectively. Cells were then electroporated with 10 μg of HCV replicon RNA pZS11 and pZS2N (Zhu et al., 2003). Two hours after electroporation, cells were pulse-labeled with Express Labeling Mix (NEN) for 2 h. HCV IRES-directed NPT II synthesis was determined with an immunoprecipitation assay (upper panel). As a control for the amount of protein loaded per lane, 1% of the cell lysates were electrophoresed in a 12.5% SDS-PAGE (lower panel). The protein band migrating with a MW of approximately 15 kDa (arrow) was present in all IFN-α-treated samples and could represent interferon-stimulated gene 15 (ISG15). (B) NPT II levels were determined with a Fuji phosphoimager and the mean values and SDs from four independent assays were plotted.

novo synthesized RNA to ribosomes and turnover of viral RNA and nonstructural proteins.

Kinetics of HCV RNA decay in cells treated with IFN-α and an RdRp inhibitor

Assembly of replication complexes and synthesis of minus strand RNA are the first steps in HCV RNA replication after translation of the polyprotein. They precede amplification of plus strand RNA. To determine which of these steps is inhibited by IFN-α, we measured the rate of decline of minus and plus strand RNA in the presence of the cytokine and the polymerase inhibitor 2CMA. In addition, we took advantage of our previous observation revealing that HCV replication in Huh7 cells, and other cell lines examined, is temperature sensitive; that is, replication was inhibited at temperatures above 37°C (Fig. 6).

The following results were obtained from the analysis of RNA levels in the presence of three different inhibitors of HCV replication: first, the rate of decline of plus strand RNA was consistently highest with IFN-α and was significantly different ($P = 0.02$) between IFN and 2CMA (Fig. 6D). Second, minus strands declined at similar rates independent of the method used for inhibition of viral replication with apparent half-lives ranging from 9.5 to 12.3 h (Fig. 6D). These half-lives also reflect the stability of viral replication complexes consisting of minus and plus strands in infected cells. Consistent with these results, the half-lives of the nonstructural proteins were approximately 10–11 h, indicating that the components of viral replication complexes turn over together at the same or very similar rates with or without IFN-α (results not shown and Pietschmann et al., 2001; Wang et al., 2003). The apparent half-lives calculated from our studies represent maximal values, because IFN-α or incubation at 39°C did not completely inhibit viral RNA synthesis (see below and Fig. 8, results not shown). Third, the rate of decline of plus and minus strand RNA was different in the presence of all three inhibitors. In the case of IFN-α and 2CMA, plus strands declined faster than minus strands (Fig. 6D). Moreover, with IFN-α, the difference between the half-lives of the two RNA strands was significant ($P = 0.02$, Fig. 6D). In contrast, minus strands declined more rapidly than plus strands at

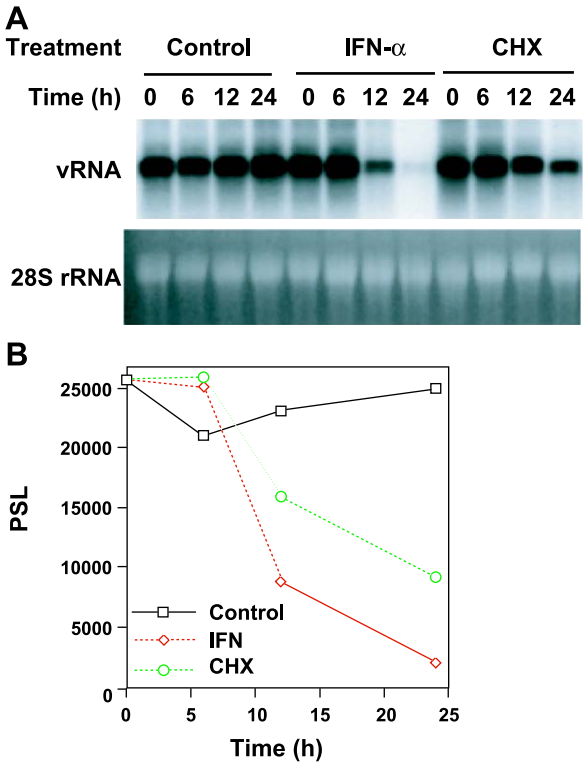

Fig. 4. Effects of IFN-α and cycloheximide on HCV RNA levels in GS4.1 cells. (A) GS4.1 cells were cultured in medium alone (control), medium containing 100 IU/ml IFN-α or 20 μg/ml of cycloheximide for the indicated time periods. Cells were then harvested and positive strand RNA levels were determined by Northern blot analysis. Ribosomal 28S RNA was used as a control for the amount of RNA loaded in each lane. (B) The amounts of HCV RNA were determined with a Fuji phosphoimager. PSL, arbitrary units.

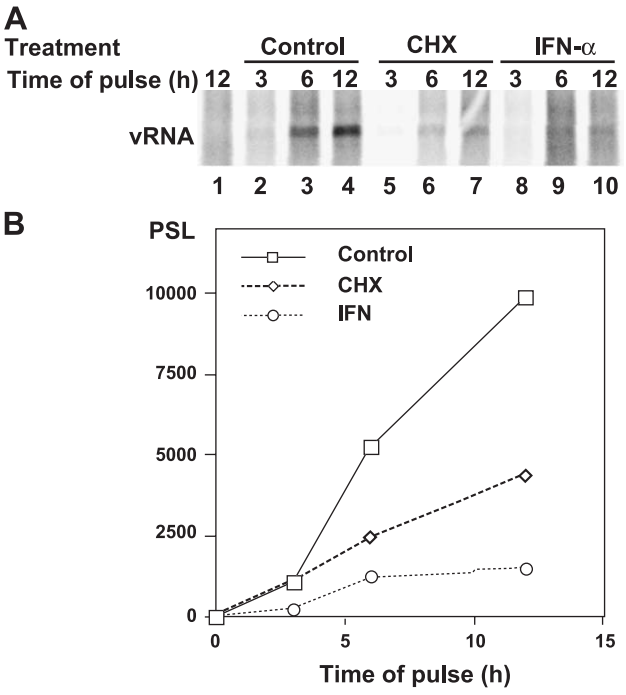

Fig. 5. Effects of cycloheximide and IFN-α on HCV RNA synthesis in GS4.1 cells. (A) Cells were left untreated (control) or treated 100 IU/ml of IFN-α for 6 h and 10 μg/ml of cycloheximide for 1 h. Cells were then radiolabeled with ^{32}P-orthophosphate in phosphate-deficient medium alone (control, IFN-α) or in the presence of 10 μg/ml of cycloheximide for 3, 6 and 12 h. Total cellular RNA was extracted and electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde. Normal Huh7 cells were pulse-labeled for 12 h to serve as negative controls. (B) The amounts of HCV RNA were determined with a Fuji phosphoimager. PSL, arbitrary units.

39°C. Fourth, consistent with these results, differences between the activities of the three inhibitors became apparent when the ratios between plus and minus strand RNAs were determined (Table 1). Consistent with the model for plus strand RNA amplification, in untreated cells, the ratios varied from 6 to 7.5. In the presence of IFN-α and 2CMA, the ratio decreased to 2.5 and 3.1, respectively, indicating that plus strands declined faster than minus strands. In contrast, complete inhibition of translation by cycloheximide did not change the ratios between plus and minus strands. Moreover, inhibition of HCV replication at 39°C resulted in an increase of the ratio above 9, indicating, as stated above, that minus strands declined faster than plus strands under these conditions. However, treatment of cells with IFN-α at 39°C seemed to reverse the ratio of the two RNA strands resulting in a pattern observed with IFN alone (Table 1). This result suggested that these inhibitors (IFN-α, 39°C) blocked HCV replication, at least in part, by distinct mechanisms.

RNA synthesis in the presence of IFN-α

Our results were consistent with a model predicting that IFN-α inhibited HCV replication in Huh7 cells by reducing viral RNA synthesis or through an increase in the turnover rate of de novo synthesized plus strand RNA, or both. To better determine the effects of IFN-α on HCV RNA replication, we measured HCV RNA synthesis in GS4.1 cells pretreated either with IFN-α for 6 h or with 2CMA by ^{32}P-orthophosphate metabolic labeling experiments. The selection of the incubation time with the cytokine was based on previous experiments showing that IFN-α elicited a complete antiviral program within 6 h of treatment. The results from this experiment showed that IFN-α appeared to inhibit RNA synthesis to nearly the same extent as 2CMA (Fig. 7). The observed inhibition of RNA synthesis by IFN-α could have been caused either directly by inhibition of de novo HCV RNA synthesis or indirectly as a consequence of accelerated degradation of newly synthesized HCV RNA. Due to the technical limitations of our experimental system, we were not able to directly measure the rate of decay of newly synthesized HCV RNA by pulse-chase labeling experiments. Instead, we determined the direct effects of IFN-α and 2CMA on viral RNA synthesis by measuring the RdRp activities in replication complexes isolated from GS4.1 cells treated with the cytokine or 2CMA for the indicated time periods. In Huh7 cells, 2CMA is rapidly converted to 2CMA triphosphate and efficiently inhibits HCV RNA synthesis by chain termination (Carroll et al., 2003).

Crude membrane fractions containing replication complexes were analyzed for HCV RdRp activity (Materials and methods). The results revealed that the RdRp activity and viral plus strand RNA (vRNA) in the extracts prepared from IFN-α treated cells declined with a similar rate, indicating that IFN-α did not directly inhibit HCV RNA synthesis. In contrast, consistent with the antiviral mechanism of 2CMA, RdRp activities in the extracts prepared from cells treated with 2CMA declined much faster than vRNA, indicating, as expected, a direct block of HCV RNA replication (Fig. 8). Interestingly, HCV NS5A, a component of HCV replication complexes, declined with similar rates in membrane fractions prepared from cells treated with IFN-α and 2CMA (Fig. 8A, lower panel), indicating that in comparison with 2CMA, IFN-α did not accelerate the turnover of HCV RNA replication complexes, essentially as described above. Our results showed that RNA synthesis continued in cells treated with IFN-α and, hence, that the antiviral program induced by the cytokine did not directly inactivate replication complexes. Such an explanation would imply that the observed decrease in HCV RNA levels induced by IFN-α in GS4.1 cells (Fig. 7) is primarily due to a decrease in the half-life of newly synthesized viral RNA.

Summary

Over 40 years have passed since the discovery of the antiviral activity of IFN-α (Isaacs and Lindenmann, 1957). Yet, the mechanisms by which IFN-induced cellular proteins inhibit viral replication are in most cases not yet completely understood. As shown in this study, the lack of an efficient

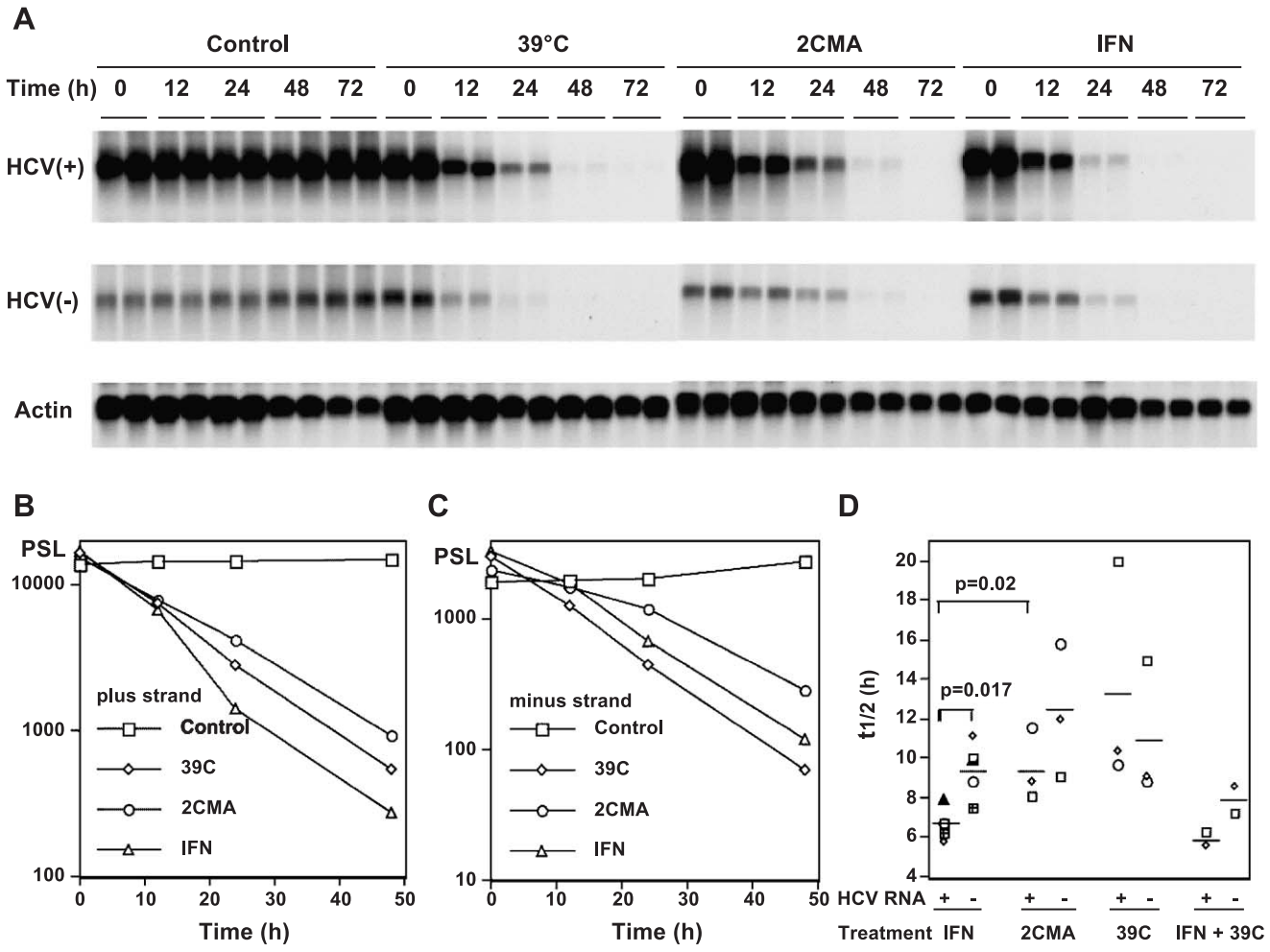

Fig. 6. Antiviral activity of IFN-α, HCV RNA polymerase inhibitor (2CMA) and heat treatment in GS4.1. (A) GS4.1 cells were treated with 100 IU/ml IFN-α, 5 μM 2CMA or cultured at 39 °C and cells were harvested before treatment (0) and 12, 24, 48 and 72 h posttreatment. Total cellular RNA was extracted with TRIzol reagent and viral plus strand and minus strand RNA levels present in GS4.1 were determined by Northern blot analysis with strand-specific probes. β-Actin mRNA served as a control for the amount of RNA loaded per lane. The amounts of HCV plus strand RNA (B) and minus strand RNA (C) were determined with a Fuji phosphoimager. PSL, arbitrary units. (D) The (apparent) half-life ($t_{1/2}$) of HCV plus (+) and minus (−) strand RNA was calculated with exponential functions obtained with a curve fitting program (CA-Cricket Graph III) and the results from two to five independent experiments were plotted. The mean values were indicated with a horizontal bar. Paired *t* tests were performed with SAS (version 8) software.

in vitro infection system and the tight linkage between translation, assembly of replication complexes and viral RNA synthesis hamper efforts to identify the viral targets. In recognizing the complexity of the problem, we sought to compare the antiviral activity of IFN-α against HCV replicons with that of inhibitors of the RdRp (2CMA), translation (cycloheximide) and presumably, assembly or stability of replication complexes (39 °C). Our results showed that the antiviral activity of the cytokine had at best a 2-fold effect on HCV IRES-directed translation that could only be detected with transfected HCV replicon RNA, but not with bicistronic reporter constructs (Figs. 2 and 3). Moreover, our results indicated that the IFN response was most likely not acting on the level of de novo RNA synthesis, although an effect on the initiation of viral plus and minus strand RNA could not be excluded (Fig. 8). Our results were consistent with the possibility that the IFN response accelerated the turnover of plus strand RNA (Figs. 6–8). If RNA degradation were involved as predicted from our model, we believe that it was not caused by the double-stranded RNA-dependent RNase L, because our previous studies showed that the vaccinia virus E3L protein, an inhibitor of double-stranded RNA-dependent activities, did not block the IFN response (Guo et al., 2003).

Our results reported here suggested that IFN-α could inhibit the replication of HCV subgenomes and full-length genomes in Huh7 cells by multiple antiviral mechanisms including inhibition of translation, acceleration of viral plus strand RNA turnover and perhaps, the formation of replication complexes. Such a model would explain why IFN-α-resistant HCV replicons could so far not be isolated in IFN-α treated cells (Guo et al., 2003).

A major question is whether the IFN-α response characterized in Huh7 cells bears any relevance for inhibition of

Table 1
Comparison of the ratios between plus and minus strand RNA in GS4.1 cells incubated with IFN-α, 2CMA at 39°C

Treatment	n[a]	Time (h)			
		0	12	24	48
Control	5	7.52 (1.58)[b]	7.42 (1.26)	7.38 (1.46)	6.78 (1.05)
IFN-α	5	6.76 (1.38)	4.50 (0.65)*	2.64 (0.62)**	2.48 (1.13)**
2CMA	3	6.03 (0.89)	4.83 (0.57)	3.80 (1.47)	3.10 (0.70)*
39°C	3	6.06 (0.55)	6.87 (1.27)	8.87 (2.38)	9.26 (1.45)*
Cycloheximide	3	7.94 (0.87)	7.88 (1.12)	7.85 (1.25)	
IFN-α + 39°C	2	6.55	2.3	1.5	1.4

[a] Number of experiments.
[b] Mean value (SD).
* $P < 0.05$ (compared with $t = 0$).
** $P < 0.01$ (compared with $t = 0$).

HCV replication in vivo. Unfortunately, a direct comparison of the kinetics of virus inhibition in vivo and in vitro is difficult, because serial liver biopsy samples cannot be obtained from IFN-treated patients. Results from microarray analyses with RNA isolated from chimpanzees during acute infections revealed a correlation between productive infection and expression of IFN-induced genes (Bigger et al., 2001). One possibility that could explain replication of HCV in the presence of an activated innate immune response is that under natural conditions, HCV replicons exhibit higher stability or replicate more efficiently compared with tissue culture systems and that the antiviral

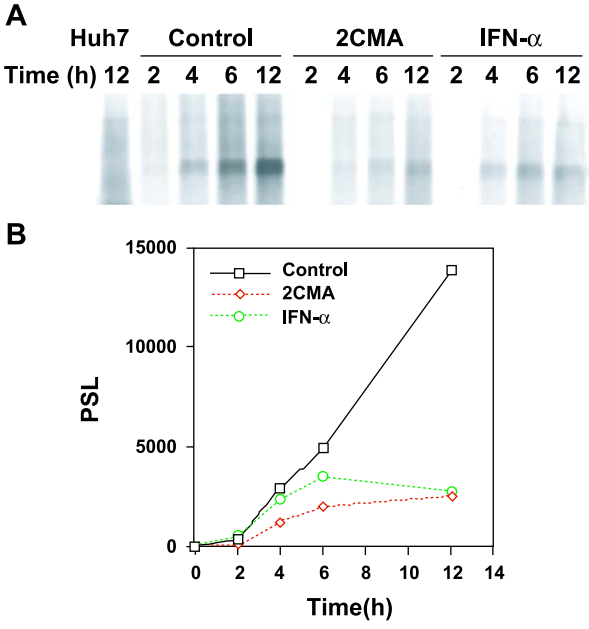

Fig. 7. Effects of IFN-α and 2CMA on HCV RNA synthesis in GS4.1 cells. (A) Cells were left untreated (control) or treated 100 IU/ml of IFN-α for 6 h and 5 μM 2CMA for 1 h. Cells were then radiolabeled with ^{32}P-orthophosphate in phosphate-deficient medium alone (control, IFN-α) or in the presence of 5 μM 2CMA for 2, 4, 6 and 12 h. Total cellular RNA were extracted and electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde. Normal Huh7 cells were pulse-labeled for 12 h to serve as negative controls. (B) The amounts of HCV RNA were determined with a Fuji phosphoimager. PSL, arbitrary units.

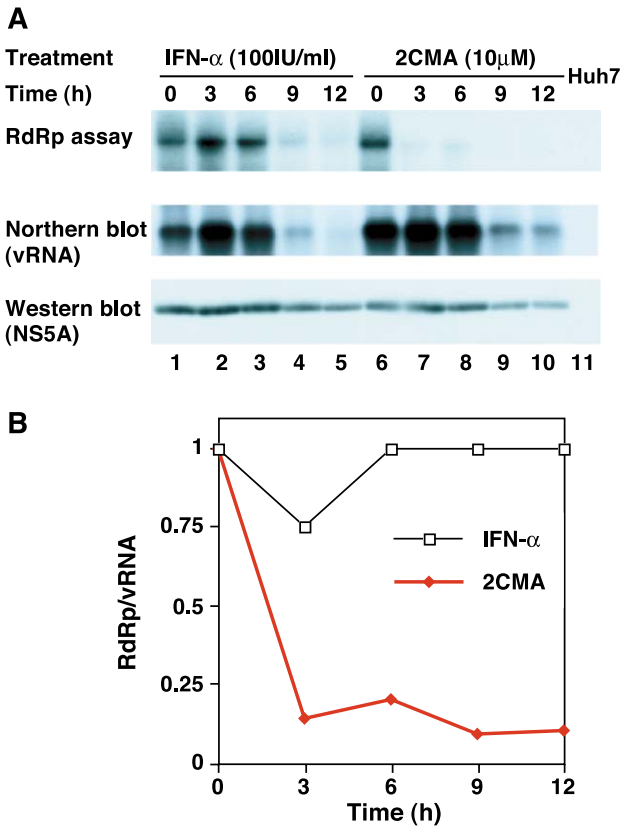

Fig. 8. Effects of IFN-α and 2CMA on HCV RdRp activity. (A) GS4.1 cells were treated with 100 IU/ml of IFN-α and 10 μM 2CMA and the cells were harvested before treatment and at 3, 6, 9 and 12 h posttreatment. Untreated Huh7 cells served as a negative control (lane 11). Crude replication complexes were prepared and subjected to an RdRp assay under the conditions described in Materials and methods. RNA products were extracted and analyzed by denaturing agarose gel electrophoresis (upper panel). HCV plus strand RNA (vRNA) and NS5A protein in the preparations of crude replication complexes were determined by Northern (middle panel) and Western blot analyses (lower panel). (B) The levels of the RdRp products and HCV RNA (vRNA) were determined with a Fuji phosphoimager. The RdRp levels were normalized to obtain an RdRp/vRNA ratio of 1 for the untreated controls shown in lanes 1 and 6.

program induced by IFN-α as described in this report functions to constrain an RNA amplification scheme designed for exponential expansion of viral RNA, as it occurs with most other positive-stranded RNA viruses. In fact, such a model would explain why HCV is a non-cytolytic virus that appears to replicate at very low levels in infected hepatocytes.

Materials and methods

Chemicals and reagents

Recombinant interferon-α 2b (Intron A) was purchased from Schering-Plough. The HCV RNA-dependent RNA polymerase (RdRp) inhibitor 2′-C-methyladenosine (2CMA) (Carroll et al., 2003) was obtained from Dr.

Joanne E. Tomassini (Merck Research Laboratories, West Point, PA).

Cell culture

The GS4.1 cell line is a subclone derived from cell line FCA1 expressing HCV subgenomic replicon I_{377}NS3-3′ (Guo et al., 2001). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin G, streptomycin, non-essential amino acids, L-glutamine and 500 μg/ml G418.

Plasmids

Plasmid pEMCVneo6 was generated as follows. pIRES-neo3 (Clontech) was digested with *Eco*RI and filled in with Klenow enzyme and then digested with *Nde*I. The vector fragment was recovered and ligated with a *Nde*I and *Mlu*I-restricted PCR fragment containing the gene for the surface antigen of duck hepatitis B virus (DHBsAg), amplified from plasmid pSP65DHBVgal5.1 (Pugh et al., 1987) using the oligonucleotide primer pairs: 5′-AAAAGCTAGCGGTGC-CAGTGATGGAAACTC-3′ and 5′-AAAAACGCGTT-TAAACTGGTAACCGAGGAATCTTAT-3′. To construct plasmid pHCVneo3, pEMCVneo6 was digested with *Pme*I and *Xba*I. The vector fragment was recovered and ligated with a *Bam*HI and *Xba*I-restricted PCR fragment containing the HCV IRES and NPT II gene, amplified from plasmid HCV-SPI (I_{377}/NS3-3′) (Guo et al., 2001) using the oligonucleotide primer pairs: 5′-TTTTGGATCCGA CTCACTATAGCCAGCC-3′ and 5′-TTTTTCTA-GAAACCGTTGTGGTCTGTTTA-3′. Because an additional 36 nt derived from the HCV core gene was fused to the 5′ end of the NPT II gene, the size of the NPT II protein expressed with pHCVneo3 was increased by12 amino acids compared to pEMCVneo6.

RNA extraction and Northern blot hybridization

Total cellular RNA was extracted with TRIzol reagent (Invitrogen). Five micrograms of total RNA was electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde and transferred onto Nylon membranes. Membranes were hybridized with riboprobes specific for plus and minus strands of HCV replicon RNA and β-actin mRNA as described previously (Guo et al., 2001).

Metabolic labeling of protein synthesis and immunoprecipitation

GS4.1 and Huh7 cells were seeded into 60-mm Petri dishes at a density of 4×10^5 cells per dish and maintained in complete DMEM. After 60 h of incubation, the cell monolayers were washed with PBS and incubated with methionine and cysteine-free DMEM medium (Sigma) for 1 h. Culture medium was replaced with 1 ml of methionine and cysteine-free DMEM medium supplemented with Express Labeling Mix (200 μCi/ml, NEN) and incubated for the indicated time periods. The medium was removed and the cells were washed with PBS and lysed with 1 ml of cell lysis buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% NP40, 1% DOC, 0.01% SDS, 1 mM PMSF, 40 μg/ml leupeptin and 100 μg/ml TPCK) in ice. The cell lysates were cleared by centrifugation at 15,000 × *g* for 5 min at 4°C. SDS and trichloroacetic acid (TCA) were added to the supernatants of cell lysates to final concentrations of 2% and 5%, respectively. The supernatants were incubated on ice for 30 min and then centrifuged at 12,000 rpm for 5 min at 4°C. Supernatants were carefully removed and pellets were dissolved in 60 μl of protein sample buffer (200 mM Tris–HCl, pH 8.8, 5 mM EDTA, 2% SDS, 1% 2-mercaptoethanol, 10% sucrose and 0.1% bromophenol blue) and boiled for 5 min and diluted with 1.2 ml of RIPA buffer (PBS supplemented with 1% NP40 and 1% DOC). For immunoprecipitation of HCV NS5A protein, 25 μl (packed volume) rabbit anti-mouse IgG pre-absorbed SPA-sepharose 4B beads was added to 0.5 ml of cell lysate and incubated for at least 2 h at 4°C with agitation, centrifuged and the supernatant was transferred into a new tube. One microgram of anti-NS5A monoclonal antibody (a gift of Dr. Chen Liu, University of Florida, Gainesville, FL) and 25 μl (packed volume) rabbit anti-mouse IgG pre-absorbed SPA-sepharose 4B beads were added into the supernatants and incubated at 4°C overnight with agitation. Beads were washed five times with RIPA buffer. The same method was used for the immunoprecipitation of NPT II, except that 25 μl rabbit anti-NPT II antibody pre-absorbed SPA-sepharose 4B beads was used. Forty microliters of 2× SDS-PAGE gel loading buffer was added and samples were boiled for 5 min. The proteins were electrophoresed through a 10% SDS-PAGE. The gels were fixed with 40% methanol and 10% glacial acetic acid for 30 min and incubated with ENHANCER solution (Amersham) for 30 min. The gels were dried and exposed to Fuji phosphoimager plates and X-ray films.

DNA and RNA transfections

Plasmid pHCVneo3 and pEMCVneo6 were transfected into Huh7 cell cells by calcium phosphate precipitation procedure (Clontech Laboratories, Inc.). Seventy-two hours posttransfection, cells were pulse-labeled with methionine and cystine-free DMEM medium supplemented with Express Labeling Mix (200 μCi/ml, NEN) for 2 h. The synthesis of DHBsAg and NPT II were determined by immunoprecipitation with a monoclonal antibody 7C12 (Guo and Pugh, 1997) and a polyclonal antibody against NPT II (US Biotech), respectively.

To determine the effects of IFN-α on HCV IRES-directed translation in the context of viral replicon RNA, Huh7 cells were mock-treated or treated with 100 IU/ml of IFN-α for 6 or12 h. The cells were trypsinized and electroporated with 10 μg of HCV replicon RNA in vitro tran-

scribed from plasmids pZS11 and ZS2N (Zhu et al., 2003) as described previously (Guo et al., 2001). After electroporation, cells were seeded onto 60-mm dishes. Two hours later, unattached cells were removed and NPT II synthesis was determined by pulse labeling with Express Labeling Mix (200 μCi/ml, NEN) for 2 h and immunoprecipitation with a polyclonal antibody against NPT II.

Metabolic labeling of HCV RNA

GS4.1 and Huh7 cells were seeded into 60-mm Petri dishes at a density of 4×10^5 cells per dish and maintained with complete DMEM. After 48 h of incubation, cell monolayers were washed with PBS and incubated with phosphate-free DMEM medium (ICN) supplemented with 5% dialyzed fetal bovine serum, 1/20th the normal concentration of phosphate and 5 μg/ml actinomycin D for 1 h. Culture medium was replaced with 1 ml of phosphate-free DMEM medium supplemented with 5% dialyzed FCS, 1/20th the normal concentration of phosphate, 5 μg/ml actinomycin D and ^{32}P-orthophosphate (200 μCi/ml, ICN, cat. number 64014) and incubated for the indicated time periods. Cells were harvested by carefully aspirating medium and washing with PBS. Total cellular RNA was extracted with TRIzol reagent (Invitrogen) and electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde. The gels were soaked in 10% glacial acetic acid for 20 min to remove acid-soluble ^{32}P and dehydrated in 95% ethanol for 20 min. The gels were dried with a gel drier at 50°C for 2–3 h and exposed to Fuji phosphoimager plates.

HCV RdRp assay

GS4.1 and Huh7 cells were treated with IFN-α and 2CMA for the indicated time periods and then washed with PBS. Cells were collected with a rubber policeman and suspended in TNMg buffer (10 mM Tris–HCl, pH 8.0, 10 mM sodium acetate and 0.5 mM $MgCl_2$) at a density of 3×10^7 cells/ml and incubated on ice for 10 min. The swollen cells were further lysed by passing through 20-gauge needle 30 times and 26-gauge needle 60 times. The cell lysate were fractionated by centrifugation at $500 \times g$ for 5 min to remove nuclei and supernatants were then centrifuged at $10{,}000 \times g$ for 10 min to pellet the membrane fraction containing HCV replication complexes. The pellet was resuspended in TNMg buffer. The reaction mixtures of HCV RdRp assay contained the following: 50 mM Tris–HCl (pH 8.0), 10 mM sodium acetate, 7.5 mM potassium acetate, 10 mM β-mercaptoethanol, 400 U/ml RNasin, 6 μg/ml actinomycin D, 0.5 mM of ATP, CTP and UTP, 25 μM GTP, 0.5 mCi/ml of α-^{32}P-GTP, 5 mM phosphopyruvate, 15 U/ml of pyruvate kinase (Sigma), 34 μl of membrane fraction from ca. 10^6 cells and H_2O to a total volume of 50 μl. Reactions were incubated at 30°C for 60 min. RNA was extracted with TRIzol reagent and electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde. The dried gels were exposed to Fuji phosphoimager plates.

Western blot analysis

Five microliters of the membrane fractions from ca. 10^5 GS4.1 or Huh 7 cells were combined with an equal volume of 2× SDS-PAGE sample buffer. The proteins were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred to Immobilon-P membranes (Millipore Corp.). The membranes were first incubated with a monoclonal antibody against HCV NS5A and then incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Amersham) and bound IgG was detected with Super-Signal chemiluminescence reagents (Pierce).

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